Nucleic Acid Probes

A Primer for Pathologists

Margaret A. Piper, PhD
Kallestad Diagnostics Research and Development
Chaska, MN

Elizabeth R. Unger, MD, PhD
Department of Pathology and Laboratory Medicine
and the Winship Cancer Center
Emory University
Atlanta, GA

ASCP Press
American Society of Clinical Pathologists
Chicago

Library of Congress Cataloging in Publication Data
Piper, Margaret, 1953-
Nucleic acid probes : a primer for pathologists / Margaret A. Piper, Elizabeth R. Unger.

Includes bibliographical references.
ISBN 0-89189-283-4
1. DNA probes—Diagnostic use—Handbooks, manuals, etc. 2. Nucleic acid probes—Diagnostics use—Handbooks, manuals, etc. I. Unger, Elizabeth R., 1951- . II. Title.
[DNLM: 1. Nucleic Acid Probes. QU 58 P665n]
RB43.8.D63P56 1989
616.07'56—dc20
DNLM/DLC
for Library of Congress. 89-17940
CIP

Printed in the United States of America.

92 91 90 89 4 3 2 1

Contents

genome copies will be present. A reliable signal may be obtained with a specific probe used in a dot/blot hybridization assay. However, if the target is an alteration in a human gene, that gene is present in only one copy per cell. The ratio of total genomic DNA to gene DNA in a cellular extract is enormous and difficult to overcome. In addition, the amount of DNA that will bind to a solid support is limited. Therefore, the amount of target DNA available for hybridization is small and the signal generated by a hybridized probe may be difficult to detect. In cases in which one probe is used to detect a change such as a deletion, both positive hybridization and the molecular weight of the restriction enzyme fragment hybridizing to the probe must be determined. Southern blotting is uniquely suited to meet these requirements.

RNA analysis by northern blotting is often used to determine if specific genes are expressed in cell or tissue samples by studying complementary mRNA, and to qualitatively determine levels of expression. Although many mRNA copies are made from an actively expressed gene, and their presence could be determined with a suitable probe and dot hybridization, northern blotting allows verification of the message size and demonstrates any problem with cross-hybridization with other mRNA species. Northern blotting can also be used to establish the size of an unknown message.

Conditions for both Southern and northern blotting are becoming more streamlined, reproducible, and suited to clinical laboratory diagnostic use, although the technique may remain in specialized reference laboratories for some time. However, optimal conditions must still be defined for each probe test to maximize the true signal-to-"noise" ratio, noise being defined as nonspecific background staining. Noise can be caused by insufficient washing stringency, inadequate prehybridization, or a probe that has been degraded. Many artifacts can be produced by this technique and their resolution requires knowledgeable interpretation and investigation. The semiautomated instruments available for electrophoresis and blotting may go a long way toward eliminating some of these procedural problems. Sensitive enzyme-amplification labeling methods may also be able to replace radioactive labels and eliminate

difficulties caused by the loss of specific radiolabel activity over time and the possible presence of autoradiographic artifacts.

Propagation of a probe in a plasmid can cause vector contamination. When the probe sequence is not completely cut and separated from the vector nucleic acid sequences, the probe material applied to the membrane for specific hybridization to test samples can nonspecifically hybridize to sequences complementary to the plasmid fragment. While appropriate controls discriminate between specific and nonspecific hybridization of the probe sequence, hybridizing vector sequences could be interpreted as new, specific bands indicating alteration of the gene in the test sample. An appropriate control for this artifact is to hybridize with vector that has not been recombined with the probe. This could be done on the same membrane by stripping the first probe and rehybridizing with digested vector. Appropriate quality control methods in large-scale production of purified probes should also prevent detectable contamination.

The Southern and northern blotting methods have as disadvantages the time required to perform the assay, as well as several steps requiring manipulation of reagents and materials. Radioactive probes require special handling and longer development times. However, these techniques offer significant advantages in that they are less subjective and less dependent on interpretive experience (as is histologic identification of neoplastic cells), and require small amounts of sample and no processing to preserve tissue morphology. In some cases, the tests also provide information that could not be obtained by any other method.

In situ hybridization

In situ hybridization is a very specialized type of solid-support hybridization represented schematically in Figure 2–7. It involves taking morphologically intact tissues, cells, or chromosomes through the hybridization process to demonstrate not only the presence of a particular piece of genetic information, but also its

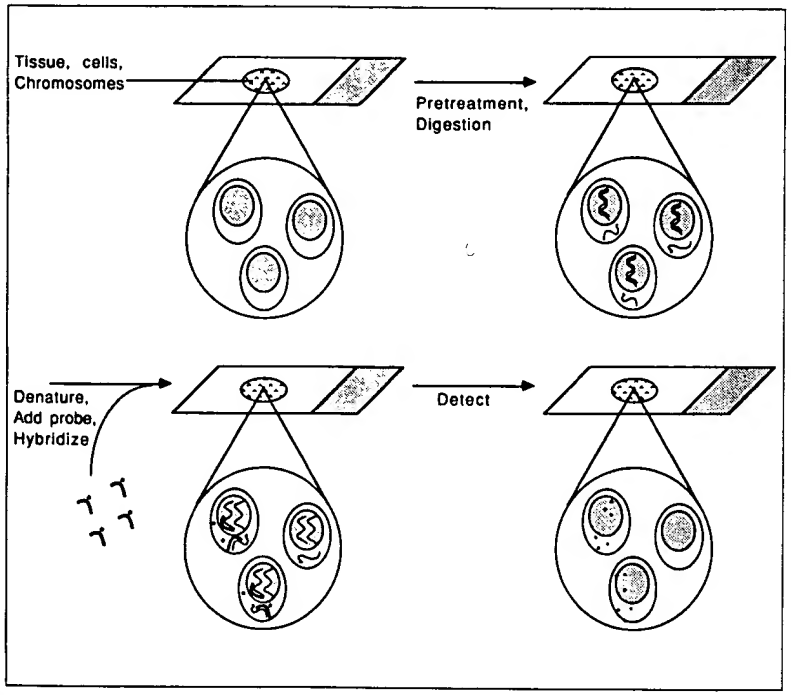

Figure 2–7. Schematic representation of in situ hybridization.

specific location within the tissue, cell, or chromosome. In situ hybridization techniques represent the best compromise between making the nucleic acid target available for hybridization and maintaining the morphologic integrity of the starting material. The target nucleic acids are found intimately mixed with the proteins, other nucleic acids, and membranes that form the basis of the familiar staining patterns with "routine" stains such as H&E, Papanicolaou, or Giemsa banding. The goal is to make the target nucleic acid available for hybridization while maintaining a recognizable "staining environment" so that the tissue, cell, or chromosome can be identified by the landmarks of routine staining.

Sample preparation involves some method of affixing the starting material to a microscopic slide, because light microscopic ex-

amination is required to evaluate the assay. The slide becomes the solid support that carries the cells, tissue, or chromosomes through all the following steps of the hybridization assay. This step is not trivial because the conditions of the hybridization assay can be quite harsh and loss of sample through detachment during the assay is a major concern. Adherence is improved by coating the glass with substances such as gelatin, polylysine, aminopropylsilane, or Elmer's Glue™.

Tissues are prepared by standard histologic methods, with perhaps some extra speed in handling to attempt to minimize degradation of sample nucleic acid by endogenous nucleases. They may be snap-frozen or fixed, routinely processed, and embedded in paraffin. Sections are then cut on a cryostat, microtome, or vibrotome and immobilized on a slide. Cells in suspension, fixed or unfixed, may be prepared for assay by cytocentrifugation or direct smearing over the surface of the slide. Alternatively, for tissue culture monolayers, the cells may be grown directly on the glass surface to be used in the assay. Chromosome preparations are "squashed" or "splattered" onto the slide to open the cells and separate the chromosomes.

The sample type and method of preparation greatly influence the details of the technique. Fresh or frozen material will behave much differently from fixed material, particularly if a cross-linking fixative such as formaldehyde or glutaraldehyde is used. Fixatives were developed to preserve morphology through routine staining procedures, and thus will add to the morphologic integrity of the finished hybridization product. At the same time, the fixatives will decrease the availability of the nucleic acid that is to undergo hybridization.

Before hybridization the sample is subjected to various enzymatic digestions to improve the accessibility of the sample's nucleic acid to the probe. Commonly a protease is used, and occasionally ribonuclease or deoxyribonuclease digestions are employed. In addition to being employed as a tissue permeabilizer, nuclease digestion can be used as a control in the procedure. If the signal is abolished by RNase treatment, the target must have

been RNA, and likewise for DNase treatment. Detergents have also been used to improve probe penetrance into the sample.

Denaturation of the sample nucleic acid is crucial to obtaining a signal after hybridization. This step is especially important when the target is DNA, which is normally present in its double-stranded helix. RNA targets are usually single-stranded, but can have secondary structure inhibiting their ability to interact with probe. Conditions of denaturation need to be slightly modified from those applicable to solution-phase reactions, especially when cross-linked fixatives are used, but the same methods (ie, heat, formamide, and base) are applicable. (Alkaline denaturation is useful only for DNA targets, since RNA is rapidly degraded under these conditions.)

The samples may be subjected to other pretreatments designed to reduce background, similar to those that membranes receive before hybridization. These include acetylation, protein blocking, and prehybridization (hybridization cocktail without probe). Hybridization occurs when the sample is covered with probe in a hybridization cocktail. If the probe requires denaturation (if it is double-stranded), it may be denatured before interaction with the sample, or the probe and sample may be denatured simultaneously by heat. The simultaneous heat denaturation of sample and probe nucleic acids may favor penetrance and minimize the tendency of tissue nucleic acid to "snap back" and reanneal with its endogenous homologue.

Hybridization cocktails and probes are identical to those used in other forms of hybridization assays. The time and temperature of the hybridization reaction are determined by the composition of the cocktail and the concentration of probe. Formamide is generally employed to allow hybridization to occur at lower temperatures, resulting in better retention of morphology. The hybridization cocktail is retained over the sample with a glass or plastic coverslip. For long hybridization times a seal of rubber cement or wax may be used. The series of graded salt washes used to remove unhybridized probe is also identical to that used in other forms of hybridization assays.

Detection of the hybridization signal depends on the method used to label the probe. Various radioactive labels may be used; all are detected with silver emulsion autoradiography. Exposure times range from overnight to several months. With low-energy radioisotopes such as tritium, there is minimal scattering of the silver grains from the site of probe localization but the required exposure times are the longest. High-energy isotopes such as ^{125}I give very poor localization of signal but require very short development times. In practice, ^{35}S is a useful compromise between signal localization and development time.

With nonradioactive labels the final detection is analogous to methods used in immunohistochemistry: either fluorescent tags or histochemical enzymes may be utilized to localize the position of the reporter molecules on the probe. There is essentially no scatter of signal with either method. The results of fluorescence may be viewed immediately, while enzyme-derived colorimetric products require 10 minutes to overnight for development. Colorimetric or fluorescent analyses are generally much faster than autoradiography, but many investigators believe there is a significant loss of sensitivity. Others feel that with careful optimization of the assay conditions, nonradioactive methods can achieve sensitivities very close to those obtained with radioactive isotopes.

After counterstaining to bring out the morphology and maximize contrast between the signal and surroundings, the final product is evaluated by light or fluorescence microscopy. Through visual inspection the presence of signal is determined qualitatively, and it is localized to a particular cell or chromosome. Autoradiographic detection can be made semiquantitative by counting the number of developed silver grains per area. Semiquantitative colorimetry can be accomplished with computer-assisted image analysis and morphometry. Attempts at quantitation can be quite complicated, and most investigators use in situ hybridization at the qualitative level.

In situ hybridization occurs and is interpreted in a morphologic context. Unlike hybridization assays based on extraction techniques, the sensitivity of the in situ assay is influenced not only

by the number of copies of the target sequence but also by its distribution. Extraction techniques are most sensitive when sequences are uniformly distributed in a sample, while in situ methods are most sensitive when target sequences are nonuniformly distributed. When only one or two cells in a relatively large sample contain the target sequences, extraction methods dilute those positive sequences, while in situ hybridization assays preserve the natural concentration and easily detect the few positive cells in a negative background. This is the only form of hybridization that combines the power of morphologic analysis with a sophisticated genetic analysis and permits definitive localization of genetic information.

The in situ hybridization assay can be quite tedious and each sample must be treated individually. This makes the handling of multiple samples very difficult and is one of the biggest drawbacks of the assay. Because morphologic integrity is crucial to the assay, investigators familiar with extraction-based techniques often find in situ hybridization to be a kind of "black magic" that is subject to many more variables influencing both signal and background. With experience in working with tissues, cells, and chromosomes, these difficulties usually can be resolved; however, the assay does have an extra level of complication compared with extraction-based techniques. The tedium of the assay is being addressed through automation. (The Code-On™ Series Slide Stainer, an instrument capable of taking samples immobilized on glass slides through all the steps of the hybridization assay, is being produced by Fisher Scientific Co. This tabletop robotic device has the potential to facilitate the introduction of this technique into more widespread routine use.)

Polymerase Chain Reaction

Recourse to use of the blotting procedures and radioactive labels is necessary in many nucleic acid probe assays to achieve maximum sensitivity. This is especially true when the gene to be detected is present only in single copies per cell, or when the amount of sample

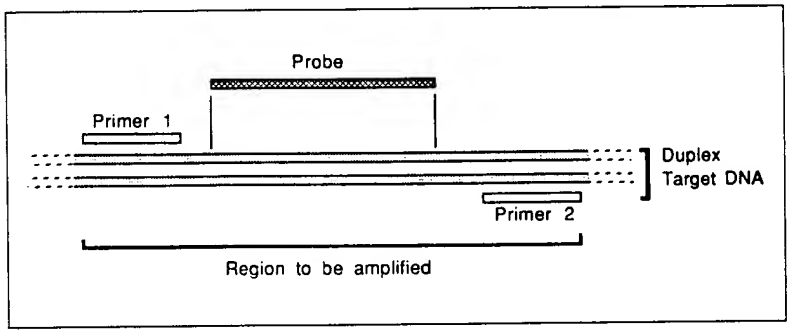

Figure 2–8. Components of the polymerase chain reaction. Two primers, specific for sequences on opposite strands, define the region of the target DNA that is to be amplified. The probe to be used for the final hybridization binds within the amplified region.

DNA that can be analyzed is very small. To circumvent these problems, a method has been developed that essentially creates more sample by amplifying specific DNA sequences for subsequent hybridization and detection. This amplification procedure, called the *polymerase chain reaction* (PCR), makes nonradioactive probe labeling without loss of sensitivity much more feasible and, in cases in which very specific probes are available, it may allow substitution of dot blotting for Southern blotting. In some cases the sequences may be amplified sufficiently to allow direct visualization of ethidium bromide–stained bands in the agarose gel.

To utilize the PCR in a particular system, it is necessary to know the base sequence of flanking portions of the gene sequence to be detected in the final hybridization reaction. With this information, small oligonucleotides are synthesized that hybridize to DNA regions on opposite strands flanking the probe target sequence. The region between and including the oligonucleotides is the region to be amplified (Figure 2–8). To begin, the genomic DNA is digested with restriction enzymes and heated to separate the complementary strands. The conditions are adjusted for hybridization and the synthetic oligonucleotides are added. The oligonucleotides act as primers for a DNA polymerase, among them